IN THE SPECIFICATION

Please amend the specification as follows.

Please replace the paragraphs at pages 4, line 5 to the last line on that page with the following rewritten text:

Figures 1A, 1B and 1C: *Mycobacterium* species produce pili. Electron micrographs showing different pili morphotypes produced by *M. tuberculosis* H37Ra (A) (x28000); *M. tuberculosis* H37Rv (B) (x25000); *M. tuberculosis* CDC1551 (C) (x22000); Arrows point to the fibers produced by the various strains tested.

Figures 2A and 2B: Electron micrographs of *M. tuberculosis* pili (Mtp) enriched extracts. A and B, Purified pili from *M. tuberculosis* H37Ra (x25000).

Figures 3A, 3B, 3C, 3D and 3E: Reaction of TB patient sera with purified Mtp. Mtp (Fig. 2A) were reacted with human TB patient sera HS14, (A), HS7 (B), HS35 (C), HS29 (D), and healthy control sera (E) and detected with anti-human IgG FITC-conjugate. Positive reactions (A-D) are depicted by the presence of fluorescent fibers. No reactivity was observed with healthy control sera (E). Magnification of fluorescence micrographs A-E x1000.

Figure 4: Sera from TB patients react to purified Mtp. Sera from TB patients (n=36) and from healthy controls (n=5) were tested for the presence of anti-Mtp antibodies by ELISA using immobilized Mtp fibers. Most of the patient sera (60%) showed a significant titer against Mtp fibers. Results presented obtained at sera 1:3200 dilutions run in triplicate. The horizontal line indicates the cut-off value of two times the average ELISA titer A_{450} reading of healthy control sera.

Figure 5: Mtp binds to extracellular matrix proteins. Shown are the results of binding of increasing concentrations of fibronectin, laminin, and collagen type IV to Mtp-coated ELISA plates. Binding was quantitated by ELISA at A_{450}. Results are presented from 3 independent experiments run in triplicate.

Figures 6A, 6B and 6C: Biofilm growth of *M. tuberculosis* H37Ra. The GFP expressing bacilli produce a film on the glass coverslip (A) shown by confocal microscopy in (B) and high resolution SEM in (C). Note the typical biofilm architecture in the low magnification reconstruction (B) and the Mtp fibers produced by the mycobacteria shown with arrow (C).

Figures 7A and 7B : Tandem mass spectroscopy fragmentation pattern of the acid hydrolysate produced from Mtp samples (A), actual identifications are indicated in red (bold) and theoretically possible fragments are black (B).

Please replace the paragraph beginning at page 12, lines 1-3, with the following rewritten paragraph:

1. Dilute 1ml frozen stock of *M. tuberculosis* cells in 10 ml 7H9 + ADC containing 0.05% TWEEN-80 ~~Tween-80~~ and incubate at 37° with shaking for 48-72 h to prepare starter culture.

Please replace the paragraph beginning at page 13, line 8 to page 14, nine lines from the bottom, with the following rewritten paragraph:

3. Initial biochemical characterization of mycobacterial pili. Generally, pilin monomers are proteins in the range of 14-25 kDa that can be identified in SDS-PAGE gels after dissociation of the pili filaments under conventional denaturing (in the presence of SDS, 2-mercaptoethanol, and boiling at 100°C) conditions. However, some bacterial pili types require special chemical treatments such as acidification (pH 1.5) and boiling with HCl, as in the case of the common *E. coli* type I pili, or denaturation with formic acid, as in the case of the *Salmonella* and *E. coli* curli fibers. We began our characterization of purified Mtp preparations in 16% SDS-polyacrylamide gels under normal denaturation conditions. After repeated efforts employing Coomasie Blue and silver staining techniques, we were unable to detect the presence of polypeptide bands in the pili samples in the range of 14 to 25 kDa that correlated with the presence of abundant pili in samples as demonstrated by TEM. The absence of other high MW polypeptide bands and TEM analysis indicated that our pili preparations were relatively pure. This suggested that Mtp were highly hydrophobic and non-dissociable under regular 2-mercaptoethanol and SDS-PAGE denaturation. In fact, we observed the presence of protein aggregates in the wells of the stacking gel confirming that the pili filaments were still intact and therefore the pilins were not entering the gel. Nevertheless, the pili fractions were subjected to N-terminal amino acid sequencing by Edman degradation. Mtp were blocked in their N-terminus. Further efforts employed several different chemical, physical and enzymatic treatments to allow dissociation of the pili

aggregates followed by denaturation of the pilin proteins by SDS-PAGE. Isolated Mtp were treated with various chemical reagents that have been used by other groups to resolve pili into their pilin monomers. Formic acid treatment was used in the manner described by Collinson *et al.*. Approximately 0.10 mg of the pili preparation was dried using speed-vacuum centrifugation, resuspended in 95% formic acid, and immediately frozen at –70°C. After 1 h, the sample was dried by heating for 96 h to remove all traces of acid. Pili were also incubated in the presence of 0.1% to10% SDS at 37°C for 18 h. Attempts were also made to dissolve the fibers in 2 to 8 M urea or saturated guanidine-HCl for 18 h at 37°C. The purified pili preparations were also incubated in 0.1, 1, and 10% TRITON-X-100 ~~Triton X-100~~ at 37°C for 18 h. The purified pili were subjected to acid (pH 1.8) or alkaline (pH 12.0) treatments and boiled for 30 min as previously described. The pili fibers were also treated with 0.5%, 1%, and 5% sodium deoxycholate and incubated at 37°C for 18 h. After the above treatments samples were either prepared for separation by SDS-PAGE or for viewing by TEM. After electrophoresis of all above treated Mtp pili samples, material was still observed in the wells of the stacking gel and no visible protein bands were detected in the separating gel following either Coomasie Blue or silver staining methods. Similar results were observed when a 10% resolving gel was also used. After all of the above chemical treatments pili fibers were still visible by TEM. These results demonstrate that *M. tuberculosis* pili are very stable and cannot be broken down into their pilin subunits by methods conventionally utilized by other research groups working with pili. Further, the purified pili from *M. tuberculosis* were treated with a variety of enzymes to determine their biochemical nature. Pepsin, trypsin, or proteinase K treatment under the appropriate enzymatic conditions and concentrations, as observed by SDS-PAGE analysis and TEM, did not degrade the pili. Due to the aggregative nature of the pili, amino acid residues of the macromolecule may not be accessible for cleavage by these proteases. Treatment of Mtp with the enzyme cellulase had no effect, demonstrating that the pili are not composed of cellulose polymers . The pili were also incubated at 37°C for 18 h with lysozyme (10 mg/ml) without effect, indicating the fibers are not polymers of peptidoglycan. The purified pili were extracted with 2:1 chloroform:methanol and found to remain in the interface after centrifugation. This indicates that the pili are not a non-polar lipid substance from the mycobacterial cell wall.

Please replace the paragraph beginning at page 16, line lines 9 to the last line on the page, with the following rewritten paragraph:

4. Detection of antibody to *M. tuberculosis* pili in sera from tuberculosis patients. An initial study was done to determine if anti-pilus antibody is present in sera from human patients with active tuberculosis infections. If such antibody could be detected, it would suggest that *M. tuberculosis* pili are produced *in vivo* during natural human infections and that they are antigenic. Toward this aim, sera from thirty-six cavitary tuberculosis patients, admitted to the Instituto Mexicano del Seguro Social, Monterrey, Mexico and sera from five healthy controls were obtained. The sera were tested against purified Mtp preparations obtained from H37Ra using immunofluorescence (IF) as previously described. Briefly, glass cover slips were prepared with a diluted pili preparation, air-dried, and fixed with PBS containing 3% formaldehyde overnight at 4°C. The coverslips were washed with PBS followed by incubation at room temperature for 1 h in sera diluted 1:1,000 in PBS containing 10% fetal calf serum (PBS/FCS). After thorough washing with PBS, to remove unbound antibody, the samples were incubated for 1 h with goat anti-human IgG Alexa Fluor 488 (Molecular Probes) diluted 1:5,000 in PBS/FCS. The coverslips were washed and mounted on glass slides before observation under a Nikon TE 2000S fluorescent microscope using Metacam software. Strikingly, we found that a high percentage (60%) of the sera from tuberculosis patients reacted very strongly with the purified pili preparation (Fig. 3A, B, C, and D). We considered the reaction positive when long fluorescent coiled fibers were observed after incubation with the patients' sera. No fluorescent filaments were observed with the goat anti-human IgG ALEXA FLUOR ~~Alexa Fluor~~ 488 alone, confirming the specificity of the reaction (data not shown). More interestingly, 5 of 5 sera from healthy human controls did not react with Mtp fibers (Fig. 3E).

Please replace the paragraph spanning pages 18 and 19, with the following rewritten paragraph:

Bacteria. Mycobacterial strains (*M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. tuberculosis* CDC1551, *M. smegmatis*) and *E. coli* strains (to be used in cloning of *M. tuberculosis* genes) will be stored as stock cultures at –70°C as thick suspensions in either Middlebrook 7H9 [mycobacteria] or LB [*E. coli*] medium containing 30% glycerol. Mycobacterial inocula for various experiments will be prepared by spreading several drops of a freshly thawed bacterial suspension to Middlebrook 7H10 agar plates which will be incubated at 37°C [three weeks for *M. tuberculosis*, three days for *M. smegmatis*] (9). Liquid cultures will be grown in Middlebrook 7H9 medium. For growth of *M. tuberculosis* H37Rv, H37Ra, and CDC1551 bacteriological media (plates and liquid) will include 10% oleic acid-albumin-dextrose-catalase supplement (OADC) and 0.05% TWEEN-80 ~~Tween-80~~ in liquid cultures only (90). The bacilli will be harvested and diluted to appropriate concentrations for use in the various studies, following procedures described above. Purity of cultures will be monitored by both acid-fast and Gram staining and by culturing on blood agar plates.

Please replace the paragraph spanning pages 22 and 23, with the following rewritten paragraph:

1. Binding of purified Mtp or pilin monomers to host cells. We will also assess the ability of purified Mtp filaments, pilin monomers, or fluorescent-beads (0.5 um, Dynamics Corp., Portland, OR) coated with these proteins and Mtp derived peptides to bind directly to cultured A549 cells, U-937 macrophages, or human macrophages. Mtp, pilin, or beads coated with these proteins, as indicated by the manufacturer's instructions, will be incubated with host cells (cultured on cover slips) for 4 h and then washed. Uncoated beads will be used as a control. The presence of fluorescent-beads will be observed directly by fluorescence microscopy, while bound pili or pilin will be detected by IF using affinity-purified anti-Mtp antibody followed by incubation with goat anti-rabbit IgG ALEXA FLUOR ~~Alexa Fluor~~ 488. Alternatively, Mtp fibers will be biotinylated with amino-sulfo-biotin (Pierce) and binding of the pili to host cells will be detected using streptavidin conjugated to ALEXA FLUOR ~~Alexa Fluor~~ 488 and

fluorescence microscopy. These experiments will provide significant information regarding the direct role of Mtp in the interaction of *M. tuberculosis* with eukaryotic cells.

Please replace the paragraph beginning at page 24, lines 13-30, with the following rewritten paragraph:

Assays to study the adherence of *M. tuberculosis* bacteria to ECM will also be done using the matrix proteins that are found to bind to Mtp by ELISA assays as described above. This bacterial adherence assay will be done following the method of Fink *et al.* (173). Twenty-four well tissue culture plates will be coated with ECM at 10 and 50 μg/ml. As controls, some wells will be untreated and others coated with BSA. Wild-type *M. tuberculosis*, pili mutant, and the complemented strains, all at 10^7 bacteria per ml in HBSS, will be added to ECM-coated wells and incubated at 37°C in a humidified CO_2 incubator for 1, 2 and 4 h. Wells will then be washed with HBSS at the various time points, bound bacteria will be recovered by treatment with one ml of 7H9 containing 1% TRITON-X-100 ~~Triton X-100~~, and viable plate counts will be determined. The percent adherence will be calculated by dividing the number of adherent CFU per well by the number of inoculated CFU. If Mtp play a role in binding to ECM, the pili mutant should bind at lower numbers than the parental or the complemented strain. To demonstrate the role of Mtp in binding to ECM in the assay, experiments will also be done using anti-Mtp antibody. If Mtp plays a role in binding to ECM, the presence of anti-Mtp in the assay will decrease the adherence of wild-type bacteria and the complemented strain to the ECM–coated wells. Alternatively, if problems arise with this methodology, ELISA plates coated with *M. tuberculosis* will be used to monitor binding of ECM as described by Marques *et al.* (172).

Please replace the paragraph spanning pages 27 and 28, with the following rewritten paragraph:

In order to determine whether Mtp vaccination stimulates a cell-mediated immune response, cytokine responses of mouse splenocytes will be determined using *ex vivo* analysis (134). Spleens from 5 mice from each study group described above will be pooled, and lymphocytes purified over a FICOLL-HYPAQUE ~~Ficoll-Hypaque~~ gradient. CD3-positive T-cells will be enriched using a T-cell enrichment column (R & D Systems) following the

procedure of Kobie *et al.* (134). Thymocytes will be re-stimulated *in vitro* using Mtp antigen or PPD pulsed bone marrow derived dendritic cells [DC] (134). These DC cultures will be prepared and matured with TNF-α as previously described (134). The DC will be primed 24 h before incubation with splenocytes by the addition of 1 or 10 μg Mtp, 1 or 10 μg of PPD (Mycos Research), or buffer alone. Controls will include DC alone and T cells alone. Supernatants will be collected after 72 h, and the levels of IFN-γ, IL-4, and IL-10 secretion will be determined by ELISA using immunoglobulin specific for mouse cytokines (PharMingen). IFN-γ cytokine production has been demonstrated to be critical in the development of a protective anti-mycobacterial immune response. Th1-type cytokines, such as IFN-γ, are critical in preventing active TB disease (122). IL-4 and IL-10 cytokine production is normally induced during a Th2- type immune response which is not optimal for control of tuberculosis infections (122, 135, 136). Thus, by monitoring the level of IFN-γ, IL-4, and IL-10 cytokine secretion in these assays we will be able to elucidate what type of cell-mediated immune response Mtp antigen induces in mice. These results will be compared to levels of cellular immunity (cytokine production) induced after TB infection of Mtp-vaccinated, BCG vaccinated, and control mice, as described above, either 7 or 14 d after aerosol challenge.